PATENT

Attorney Docket No. 3299.2

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant: Fred Christians et al.

Serial No: Unassigned

Filing Date: Herewith

Title: **Preparation of Nucleic Acid Samples**

Examiner: TBD

Group Art Unit: TBD

Commissioner for Patents
Washington, D.C. 20231

**PRELIMINARY AMENDMENT**

Dear Sir:

Prior to examination of the above-referenced application, please amend the accompanying application as follows:

IN THE SPECIFICATION

Please replace paragraph on page 2, line 5 with the following:

This is a divisional application of U.S. Serial No. 09/689,937 filed October 11, 2000 which claims the benefit of U.S. Provisional Application No. 60/162,739 filed October 30, 1999 and U.S. Provisional Application No. 60/191,345 filed March 22, 2000.

Paragraph beginning on page 20 line 20 with the following paragraph:

The following procedure was performed in PCR tubes in a thermocycler. An initial mixture was prepared by mixing 25 µ÷g of total *E. coli* RNA to 13.75 µ÷L of 5.0 µ÷M rRNA Reverse Transcriptase (RT) Primer Mix, and adding deionized water (DI $H_2O$) to a final volume of 30 :L and a concentration of .83 µ÷g/ µ÷L of RNA.

Paragraph beginning on page 20 line 24:

The following primers were used to target 16S and 23S RNA (each primer is 5 µM in the RT primer mix):

16S1514 5'-CCTACGGTTACCTTGTT-3'

16S889 5'-TTAACCTTGCGGCCGTACTC-3'

16S541 5'-TCGATTAACGCTTGCACCC-3'

23S2878 5'-CCTCACGGTTCATTAGT-3'

23SEco2064 5'-CTATAGTAAAGGTTCACGGG-3'

23SEco1519 5'-TCGTCATCACGCCTCAGCCT-3'

23S1012 5'-TCCCACATCGTTTCCCAC-3'

23S539 5'-CCATTATACAAAAGGTAC-3'

Paragraph beginning on page 21 line 7:

To the above mixture, a reverse transcription mixture including 10 µL of 10X MMLV RT Buffer, 5 µL of 100mM DTT, 2:L of 25mM dNTP Mix, 3 µL of 24.5U/µL RNAse Inhibitor (RNAguard Ribonuclease Inhibitor (Porcine), Amersham Pharmacia Biotech, P/N 27-0816-01), 6 µL 50U/µg MMLV Reverse Transcriptase (Epicentre Technologies, P/N MCR85101) and 44 µL of DI $H_2O$ was added and the reaction was carried out at 42°C for 25 minutes and transferred to 45°C for an additional 20 minutes. The mixture was then transferred to 4°C.

Paragraph beginning on page 21 line 14:

The rRNA in the DNA:RNA hybrids was then digested by adding 5 µL of 10U/µL RNAse H (Epicentre Technologies, P/N R0601K) at 37 C for 45 minutes. The enzyme was heat deactivated at 65°C for 5 minutes and then transferred to 4°C.

Paragraph beginning on page 21 line 17:

The DNA was then removed by adding 2.5 μL of 5U/ul DNAse I (Amersham-Pharmacia Biotech P/N 27-0514-01) and 1 μL of 24.5U/ μL RNAse inhibitor. Digestion was carried out at 37°C for 20 minutes and the enzyme was deactivated by adding EDTA to a final concentration of 10mM.

Paragraph beginning on page 21 line 28:

The removal efficiency for 16s and 23s rRNA is typically between 80-90%. Figures 6 and 7 show ~~shows~~ the results of hybridization of enriched and non-enriched RNA to microarrays. Fig. 6 shows hybridization of labeled unenriched RNA to a microarray. Fig. 7 shows hybridization of labeled enriched RNA to an identical microarray. As can be seen by comparing Figs. 6 and 7, the hybridization in Fig 7 shows a much cleaner hybridization with less signal produced by cross hybridization.

Paragraph beginning on page 22 line 8:

Cloned DNAs encoding the *E. coli* 16S and 23S rRNA genes were amplified separately by PCR and purified with the QIAquick PCR purification kit (QIAGEN P/N 28104). One μg of 16S and 1 μg of 23S rDNA were combined in a PCR tube and diluted to 25 μL with DI $H_2O$. The DNA was denatured by heating at 99°C for 5 minutes in a thermocycler. The tube was transferred to 70°C followed by the addition of 25 μL of a prewarmed (at 70°C) solution containing 1 μg *E. coli* total RNA, 200 mM NaCl, 100 mM Tris (pH 7.5). The tube was incubated at 70°C for 30 minutes to permit annealing of the rRNAs to the corresponding complementary strand of rDNA (approximately 1:1 molar ratio). The tube was then transferred to 37°C followed by the addition of 50 μL of a prewarmed (at 37 C) solution containing 2 units of *E. coli* RNAseH (Epicentre Technologies P/N R0601K), 50mM Tris (pH 7.5), 100mM NaCl, 20mM $MgCl_2$, and the reaction was incubated at 37°C for 20 minutes to digest RNA from DNA:RNA hybrids. DNA was then digested by the addition of 2 units of DNAse I (Epicentre Technologies, P/N D9902K) and incubation at 37°C for 15 minutes. EDTA was then added to a final concentration of 20 mM to inhibit further nuclease activity.

RNA was purified with an RNeasy column (QIAGEN P/N 74104) and then analyzed in a denaturing agarose gel stained with ethidium bromide.

Paragraph beginning on page 23 line 4:

Cloned DNAs encoding the *E. coli* 16S and 23S rRNA genes were amplified separately by PCR and purified with the QIAquick PCR purification kit (QIAGEN P/N 28104). 0.6 μg of 16S and 0.6 μg of 23S rDNA were combined in a PCR tube and diluted to 48 μL with DI $H_2O$. The DNA was denatured by heating at 99°C for 5 minutes in a thermocycler. The temperature was lowered to 70°C followed by the addition of 48 μL of a prewarmed (at 70°C) solution containing 6 μg *E. coli* total RNA, 200 mM NaCl, 100 mM Tris (pH 7.5), and 12 units of thermostable RNAse H (Epicentre Technologies, P/N H39100). The tube was incubated at 70°C for 1 minute to permit annealing of the rRNAs to the corresponding complementary strand of rDNA (approximately 1 mole DNA per 10 moles RNA). The temperature was reduced to 50°C for 5 minutes to complete one cycle of enrichment. The temperature was then increased to 70°C for 1 minute then again reduced to 50°C for 5 minutes to complete the second cycle. This temperature cycling was repeated a total of 30 times. After 1, 5, 10, 20, and 30 cycles 16 μL (corresponding to 1 μg RNA from the starting mixture) was removed from the tube and mixed with 1 unit DNAse I (Epicentre Technologies, P/N D9902K) and incubated at 37°C for 15 minutes. EDTA was then added to a final concentration of 20 mM to inhibit further nuclease activity. RNA was purified from each sample with an RNeasy column (QIAGEN P/N 74104) and then analyzed in a denaturing agarose gel, along with 1 μg of untreated *E. coli* total RNA (Figure 9). The diminishing amounts of 23S and 16S RNA as cycles are repeated can be seen by comparing the lanes from left to right. The first lane (labeled U) is untreated. The next lanes are the amount of 23S and 16S RNA after 1, 5, 10, 20 and 30 cycles, respectively.